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Carcinogen Metabolism in Human Lung Tissues and the Effect of Tobacco Smoking: Results from a Case–Control Multicenter Study on Lung Cancer Patients

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Cigarette smoking is the strongest risk factor for lung cancer, but genetically determined variations in the activities of pulmonary enzyme that metabolize tobacco-derived carcinogens may affect individual risk. To investigate whether these enzymes (e.g., *CYP1A*-related) can serve as markers for carcinogen–DNA damage, lung tissue specimens were taken during surgery from middle-aged men with either lung cancer or non-neoplastic lung disease. Phase I [aryl hydrocarbon hydroxylase (AHH), ethoxycoumarin *O*-deethylase (ECOD)] and phase-II (epoxide hydrolase, UDP-glucuronosyltransferase, glutathione *S*-transferase) enzyme activities, glutathione and malondialdehyde contents were determined in lung parenchyma and/or bronchial tissues; some samples were also analyzed for DNA adducts, using ^{32}P-postlabeling. The data were then analyzed for the following: *a*) differences in metabolic profiles between bronchial and parenchymal lung tissue; *b*) the effect of recent exposure to tobacco smoke on enzyme inducibility and benzo[*a*]pyrene metabolism; *c*) differences in enzyme inducibility between lung cancer and non-lung cancer patients; *d*) the effect of smoking on metabolism of mutagens *in vitro*; *e*) pulmonary DNA adduct levels and AHH activity in lung parenchyma of smokers and ex-smokers; *f*) lipid peroxidation products in lung tissue from lung cancer and non-lung cancer patients, as related to smoking habits and degree of airway obstruction; and *g*) prognostic value of AHH pulmonary activity in lung cancer patients. The results demonstrate a pronounced effect of tobacco smoke on pulmonary metabolism of xenobiotics and prooxidant state and suggest the existence of a metabolic phenotype at higher risk for tobacco-associated lung cancer.

Introduction

Cigarette smoking is the strongest risk factor for lung cancer so far identified (*1*), but the variation in individual cancer risk suggests that host factors, either acquired or inherited, play a role (*2–5*).

Inducibility of aryl hydrocarbon hydroxylase (AHH) activity in mitogen-stimulated lymphocytes has previously been associated with a higher risk of lung cancer in smokers (*6–8*).

In 1982, we started to test the hypothesis that the activity of pulmonary enzymes such as AHH (which reflect mainly cytochrome P450 IA1-mediated reactions) and other drug-metabolizing enzymes, known to be partly under genetic control, reflects the rates of metabolic activation and inactivation of tobacco-related carcinogens and could therefore serve as a marker for the extent of carcinogen-derived DNA damage that accumulates in the lungs of smokers. In the past, there have been methodological problems with the lymphocyte AHH-induction assay in which individual enzyme (AHH) activity was generally tested in nontarget cells, such as circulating lymphocytes. We therefore set up a multicenter study (involving the University of Pisa, Italy; the University of Genoa, Italy; and the International Agency for Research on Cancer, Lyon, France) to relate interindividual variations in pulmonary carcinogen metabolism, measured in lung samples freshly obtained at surgery from lung cancer and noncancer patients, to cancer susceptibility.

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This article briefly summarizes the major findings of the case–control study, which have been presented in detail together with a more complete bibliography elsewhere (*9–15*).

Subjects and Methods

Study Subjects

If not stated otherwise, study subjects were male patients undergoing surgery for lung cancer ($n = 60$) or other thoracic disease ($n = 20$) at the University Hospital, Pisa, Italy. None of the patients were receiving drugs known to be human enzyme inducers, and none of the cancer patients had received prior X-ray treatment or chemotherapy. Data were collected regarding pulmonary and/or extrapulmonary diseases and smoking habits. Patients were divided into smokers, ex-smokers and nonsmokers; the latter two were defined as subjects who had refrained from smoking for more than 6 months, and had never smoked, respectively. Due to limitations in the amount of tissue obtained, not all end points were investigated for every subject, and results from subsets are described in the text.

Preparation of Lung Postmitochondrial (S-12) Fractions

Specimens of peripheral lung parenchyma and, for a subset of patients, bronchial tissue samples were obtained at surgery from each patient. In lung cancer patients, tissue was taken from a location as distant as possible from the tumor. Each specimen was kept in cold 0.15 M KCl solution until homogenization, which was always carried out within 1–2 hr after surgery and S-12 fractions were prepared as described previously (*10*). For some studies, lung microsomes were prepared according to standard procedures.

Determination of Enzyme Activities

Enzyme activities were determined as described by Petruzzelli et al. (*10*). AHH, ethoxycoumarin *O*-deethylase (ECOD), epoxide hydrolase (EH), UDP-glucuronosyltransferase (UGT), and glutathione *S*-transferase (GST) activities and glutathione (GSH) and malondialdehyde (MDA) contents were measured in duplicate in coded samples. MDA, an indicator of lipid peroxidation, was determined as described elsewhere (*16*). For some lung samples, the microsomal conversion of (−)benzo[*a*]pyrene (BP)-7,8-diol into tetrols was quantified using high-performance liquid chromatography and fluorescence detection.

Mutagenicity Assays

The efficiency of lung S-12 fractions in activating promutagens or in decreasing the mutagenicity of directly acting mutagens was investigated in the Ames reversion test, as described by De Flora et al. (*9*).

^{32}P-Postlabeling

DNA adducts were isolated by ^{32}P-postlabeling assay after enrichment of adducted nucleotides, using dephosphorylation of normal nucleotides with nuclease P_1, chromatographic clean-up, and transfer chromatography and separation, as described by Geneste et al. (*14*). Normal nucleotides and adducts were quantified using liquid scintillation counting. The adduct level was calculated on the basis of the assumption that the adducts were completely resistant to 3′-dephosphorylation by nuclease P_1.

Airway Obstruction

This was assessed by flow-volume spirometry curves. The results were expressed as a percentage of the predicted values derived from a general population sample in Italy (*17*).

Statistical Analysis

If not otherwise stated, conventional methods of one-way analysis of variance were used for comparisons between groups (with the use of covariates when appropriate), and simple bivariate linear regression methods were used for correlation analysis. Data were transformed logarithmically whenever required, to normalize their distribution.

Results and Discussion

Characteristics of Study Subjects

Lung cancer patients (54.5 years of mean age) were significantly older ($p < 0.001$) than noncancer patients (45.7 years) and the mean number of pack-years (corresponding to the number of cigarettes smoked per day per year of smoking/20) was significantly higher (48.5 ± 26.3 *vs.* 27.7 ± 21.6; $p < 0.01$) in cancer patients than in noncancer patients. Of the lung cancer cases, 25 had squamous-cell carcinoma, 16 had adenocarcinomas, 9 had undifferentiated large-cell carcinomas and 2 had undifferentiated small-cell carcinomas. The cancer-free controls had miscellaneous pathological conditions, as described previously (*10*).

Comparison of Phase I and II Drug-metabolizing Enzymes in Human Bronchial and Lung Parenchymal Tissue

Pulmonary drug metabolism was investigated by measuring the activities and levels of AHH, EH, GST, UGT and GSH in S-12 fractions of bronchial tree and peripheral lung parenchyma from 21 patients, all smokers or ex-smokers (*11*). When compared to bronchial preparations, lung parenchyma contained considerably higher concentrations of reduced GSH and significantly higher EH activity. Enzyme activities in peripheral lung and bronchial preparations from the same patient (a subset of 10) were correlated positively (AHH: $r = 0.77$, $p < 0.009$); GST: $r = 0.83, p < 0.01$; EH: $r = 0.63, p = 0.07$; UDPGT:

$r = 0.73$, $p < 0.1$). These results indicate that there is a common genetic control of these enzymes in different cells and tissues from one individual.

As comparisons of parenchymal and bronchial tissue from the same patient (either cancer or noncancer) revealed no statistically significant difference (except in EH activity and for GSH level), lung parenchymal tissue specimens were analyzed further in detail.

Differential Pulmonary Enzyme Induction in Cancer and Noncancer Patients and the Effect of Smoking

AHH, ECOD, EH, GST, and UGT activities and GSH and MDA contents were determined in S-12 fractions from nontumorous parenchymal tissues taken during surgery from middle-aged men with ($n = 54$) or without ($n = 20$) lung cancer (*10*). Interindividual differences in enzyme activities ranged from 11- to 440-fold, and GSH content varied by 30-fold. The values showed unimodal distributions (all subjects). AHH, ECOD, EH, and UGT activities were significantly and positively correlated with each other implying similar regulatory control of their expression (Table 1). No difference was found in enzyme activities or GSH content between cancer and non-cancer patients or between smokers and nonsmokers. When the number of days since stopping smoking was considered, however, a significant increase in AHH, EH, and UGT activities and a smaller, but significant, decrease in GST activity were found in smokers, as compared to nonsmokers. The activities of these enzymes returned to the level found in nonsmokers within several weeks, revealing a long-lasting effect of smoking. Cancer patients who had been recent smokers (within 30 days before surgery) had significantly ($p < 0.01$) induced AHH and ECOD activities (mean: about 2- and 7-fold, respectively) when compared with smoking noncancer patients (Fig. 1).

In a subsequent study the inducing effect of recent smoking on carcinogen metabolism in the lung was confirmed in another group of lung cancer patients (obtained from the University Central Hospital, Helsinki, Finland). Metabolism of BP by lung tissues *in vitro* was measured by analyzing the metabolic products of (−)BP-7,8-diol, i.e., BP-tetrols (*18*). The stereoselective activation of BP has been implicated as an important factor in the carcinogenesis of (+)*anti*-BP-diol epoxide [(+)*r*-7,*t*-8-dihydroxy-*t*-9,10 oxy-7,8,9,10-tetrahydro-BP], the oxidation product of (−)BP-7,8-diol. BP-*anti*-tetrols, mostly consisting of *r*-7,*c*-10,*t*-8,*t*-9-tetrahydroxy-7,8,9,10-tetrahydro-BP, were measured by HPLC/fluorescence detection (*19*) after incubation with lung microsomes from lung cancer patients (smokers, ex-smokers and nonsmokers). Recent smokers had significantly (4- to 7-fold) higher metabolic activity, expressed as picomole tetrols/hr/mg protein, than ex-smokers and nonsmokers. Pulmonary AHH activity was correlated with tetrol formation in the same lung microsomal sample ($r = 0.67$, $p < 0.01$).

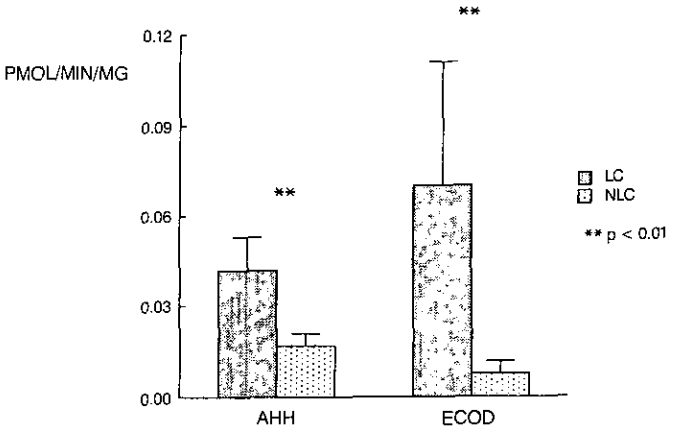

FIGURE 1. Enzyme induction of pulmonary aryl hydrocarbon hydroxylase (AHH) and ethoxycoumarin *O*-deethylase (ECOD) activities in lung cancer patients (LC) who were recent smokers (within 30 days before surgery), when compared with noncancer patients who smoked (NLC). Data extracted from Petruzzelli et al. (*10*); mean (± SEM) are plotted.

In another sub-group of lung cancer patients ($n = 25$; from University Central Hospital, Helsinki, Finland), pulmonary AHH activity also showed a good correlation ($r = 0.59$; $p < 0.01$) with the intensity of immunohistochemical staining for cytochrome P450IA by a monoclonal antibody (raised against 3-methylcholanthrene-inducible rat cytochrome P450) in human lung tissue sections. Smoking and peripheral types of lung cancer were positively related to high levels of pulmonary cytochrome P450IA, probably reflecting high rates of induction by smoking (*20*).

These results are supported by independent findings: AHH activity in normal human cells induced with polycyclic aromatic hydrocarbons was closely correlated with the expression of the structural *CYP1A1* gene (*21*). Active smoking has been found to be correlated positively with the expression of CYP1A1 mRNA in human lung tissue, and the gene expression was no longer detected 6 weeks after stopping smoking (*22*).

The results reinforce previous circumstantial evidence from studies of AHH induction in lymphocytes (see Introduction) that differential inducibility of pulmonary

Table 1. Relationships among human lung parenchymal AHH, ECOD, EH, UGT, and GST activities.[a]

	Correlation coefficient			
	ECOD	EH	UGT	GST
AHH	0.50* (45)[b]	0.48* (60)	0.28† (59)	−0.55 (60)
ECOD		0.43‡ (43)	0.48* (39)	−0.27[c] (43)
EH			0.44* (63)	−0.25‡ (69)
UGT				−0.22 (63)

Abbreviations: AHH, aryl hydrocarbon hydroxylase; ECOD, ethoxycoumarin *O*-deethylase; EH, epoxide hydrolase; UGT, UDP-glucuronosyltransferase; GST, glutathione *S*-transferase

[a]Data from Petruzzelli et al. (*10*).

[b]Number of determinations available.

*$p < 0.001$.

†$p < 0.05$.

‡$p < 0.01$.

enzymes (*CYP1A1* expression) in tobacco smokers is related to lung cancer risk in smokers.

Pulmonary Metabolism of Mutagens As Related to Drug-metabolizing Enzyme Activities and GSH Content in Lung Parenchyma

The S-12 fractions of lung peripheral parenchyma obtained from lung cancer ($n = 60$) and noncancer patients ($n = 20$) were assayed for their ability either to convert pro-mutagens into bacterial mutagens or to decrease the activity of directly-acting mutagens in the Ames reversion test (*9*). In this assay, S-12 preparations were ineffective in activating *N*-nitrosomorpholine, 3-methylcholanthrene, BP or BP-7,8-diol. Borderline, sporadic activation was seen with cigarette smoke condensate and weak but frequent activation with 2-aminofluorene, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline and cyclophosphamide. Pulmonary metabolism consistently decreased the mutagenicity of directly-acting mutagens, sodium dichromate, the acridine and nitrogen mustard derivative ICR 191, epichlorohydrin, and 4-nitroquinoline *N*-oxide. The mutagenicity of the same compounds was also decreased, although less efficiently, by the corresponding preparations of bronchial tree, as assessed with 26 specimens (*23*). In general, smoking habits were associated with stimulation of detoxifying mechanisms for some of the directly-acting mutagens investigated.

Significant correlations were seen between several biochemical and enzymic parameters and mutagenicity when evaluated in the same lung specimens (*15*).

Correlation between Pulmonary DNA Adduct Level and AHH Activity in Lung Parenchyma of Smokers and Ex-smokers

In order to test the hypothesis that pulmonary drug-metabolizing enzymes (expressed by a particular metabolic phenotype or genotype) reflect the rate of metabolic activation or inactivation of tobacco-related carcinogens in the lungs of smokers, and thus can serve as markers for the internal dose of DNA-reactive metabolites generated in target tissue, we determined the level of DNA adducts in lung parenchyma of smokers and whether these are correlated with AHH activity in the same tissue. The number of DNA adducts per 10^8 nucleotides was determined by scintillation counting after ^{32}P-postlabeling analysis (*14,24*). Microsomal fractions of the same lung specimen were assayed for AHH activity by a fluorimetric method. Smokers had significantly higher levels of DNA adducts per 10^8 nucleotides (mean ± SD: 5.38 ± 3.19) than ex-smokers (1.09 ± 0.84). AHH activity (pmole 3-HO-BP/min/mg protein) was significantly higher ($p < 0.05$) in recent smokers (0.26 ± 0.26) who smoked until 1 week before surgery than in those who had stopped smoking for more than 7 days (0.11 ± 0.11). A positive, linear correlation between DNA adduct levels and AHH activity ($r = 0.69$; $p = 0.001$; $n = 19$) was found in smokers. Such a relationship, if generally true (keeping in mind the limitations of the ^{32}P-postlabeling assay and the small number of subjects), could explain why AHH activity or inducibility expressed by certain metabolic phenotypes or genotypes appears to be a crude marker for lung cancer risk in smokers, as seen in earlier studies (see "Introduction"). The extent of DNA damage generated in target cells of a smoker's lung would be expected to differ between the "AHH-inducible" and "noninducible" phenotype. Although this remains to be proven, we have found that the (mean) levels of pulmonary AHH and ECOD were significantly higher (about 2- to 7-fold) in lung cancer patients who had smoked within 30 days before surgery than in cancer-free subjects with a similar smoking history (*10*).

Pulmonary Lipid Peroxidation Products in Cigarette Smokers and Lung Cancer Patients

Cigarette smoke may also enhance lung carcinogenesis by free radical-mediated reactions. Such radicals are present in smoke and can attack DNA directly or cause membrane damage, and they can activate oxygen (*25*), a process that has been associated with tumor promotion. We therefore studied lipid peroxidation in lung tissues from a subset of lung cancer and noncancer patients and its relationships with smoking habits and degree of airway obstruction (*12*). Specimens of peripheral lung parenchyma, free of tumorous tissue, were taken, and the (MDA) content was measured in the S-12 fractions. Airway obstruction was assessed by flow-volume curves, and data were expressed as percentage of the predicted values. Cigarettes smoked were expressed as pack-years. Lung cancer patients and controls did not differ with regard to MDA content, age, or number of pack-years. $FEE_{75,85}$ (forced expiratory flow between 75 and 85% of forced vital capacity) and MEF_{75} (maximal expiratory flow at 75% of forced vital capacity) were significantly lower in cancer than in noncancer patients ($p = 0.05$). MDA content was inversely correlated with the number of days patients had refrained from smoking ($r = 0.66$, $p < 0.01$), and was higher in recent smokers (i.e., people who had smoked during the last 30 days before surgery) than in the other patients ($p < 0.05$). Among the recent smokers, MDA content was higher in cancer patients than in noncancer patients ($p = 0.059$). When patients were divided into "high MDA" and "low MDA" groups, MEF_{75} was much lower in the high MDA group than in the low MDA group ($p < 0.01$). These results suggest that an enhanced level of prooxidant state in the lungs is associated with recent cigarette smoking; *b*) cancer patients are more prone than noncancer patients to oxidative stress; *c*) MDA level and degree of small airway obstruction were associated and differed between cancer and noncancer patients, even though these groups did not differ in the percentage of recent smokers; and *d*) a common free-radical-mediated pathway may contribute to both lung cancer induction and small airway obstruction.

Prognostic Value of Pulmonary AHH and EH Activities in Patients with Tobacco-related Lung Cancer

We have conducted an analysis on use of pulmonary drug-metabolizing enzymes (AHH, EH) as prognostic markers in male lung cancer patients. A subset of 50 lung cancer patients who had undergone thoracic sugery was used. The activity of parenchymal AHH and EH in homogenates of nonneoplastic surgical lung specimens (see previous section) was compared with the patients' survival after surgery. When the crude mortality percentages at 1 and 2 years were calculated in relation to AHH and EH activity, subdivided into quarters of the distribution, higher mortality was related to higher enzyme activities (Table 2). Subjects in the first and fourth quarters of the distribution showed significant differences in 1-year survival in relation to AHH ($p = 0.05$) and EH ($p < 0.01$) activities. This relationship could not be accounted for by age, cumulative lifetime smoking, recent or continuing smoking, stage, or histological type of disease.

The molecular mechanisms by which AHH or EH may be related to the prognosis of lung cancer patients remain obscure. Both enzyme activities can be induced by tobacco smoke, and they are positively correlated with each other ($r = 0.47$, $p < 0.001$). This could explain, at least in part, the concordance of results found here for their prognostic value; however, our data are in agreement with those on the prognostic value of intratumoral AHH activity in breast cancer patients (*26*), although the etiology and pathophysiology of these tumors are certainly different. It is conceivable that in lung cancer patients higher AHH activity would reflect a high total body burden of tobacco-related carcinogens, including enzyme-inducing substances that bind to the *Ah* receptor in lung tissue. Such substances are easily stored in adipose tissue, are deposited in the lung, and might suppress immunological functions, possibly leading to easier metastasis and faster growth of tumors. It is also plausible that the *Ah* receptor controls not only *CYP1A1* expression (AHH activity) but also gene functions unrelated to drug metabolism which are involved in growth regulation and tumor promotion (*27*). In addition or alternatively, aromatic hydrocarbons and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-like substances reported to be present in tobacco smoke (*28*) may not only bind to the *Ah* receptor but could also interact with other cell growth-controlling receptors (e.g., estrogen, epidermal growth factor and glucocorticoid receptors or other TCDD-responsive genes) leading to faster outgrowth between lung tumors.

Table 2. Mortality of lung cancer patients 1 and 2 years after surgery according to their pulmonary AHH and EH activity.[a]

	Enzyme activity (distribution)	
	1st Quarter	4th Quarter
AHH activity, pmole/min/mg, protein	0–0.006	0.039–0.280
Number of patients	12	11
Deceased after 1 year, %*	8	55
Deceased after 2 years, %†	25	73
EH activity, nmole/min/mg, protein	0.05–0.121	0.251–0.540
Number of patients	12	12
Deceased after 1 year, %‡	0	58
Deceased after 2 years, %§	25	75

[a]From Bartsch et al. (*13*). Symbols denote significance of the difference in mortality between subjects in the 1st and 4th quarters of the distribution (chi-square with Yate's correction).
*Chi-square = 3.81, $p = 0.05$.
†Chi-square = 3.50, $p < 0.10$.
‡Chi-square = 7.26, $p < 0.01$.
§Chi-square = 4.17, $p < 0.05$.

Taken together, our results reinforce previous circumstantial evidence from lymphocyte assays that *CYP1A1* expression (inducibility of pulmonary AHH) in tobacco smokers is associated with cancer risk. Our preliminary results on the positive relationship between pulmonary DNA adducts and AHH activity suggest a mechanistic basis for the difference in risk between certain metabolic pheno- and genotypes for tobacco-induced lung cancer: Among smokers with the same cigarette consumption, the AHH-inducible phenotype would be predicted to generate a higher amount of certain carcinogen–DNA damage in target cells of the lungs, a hypothesis that remains to be confirmed.

Enzyme induction by smoke constituents would be expected to increase the formation of both reactive and inactive metabolites in smokers' lung. However, it has been suggested that CYP1A-mediated insertion of oxygen into conformationally hindered positions in planar substrates, such as polycyclic aromatic hydrocarbons and aromatic amines, results in the formation of reactive intermediates which are poor substrates for subsequent conjugation and detoxication (*29*). In contrast, oxygenation at conformationally unhindered positions of chemicals by other cytochromes (e.g., CYP2B) is followed by rapid conjugation that results in detoxication. It is therefore likely that certain tobacco smoke-derived carcinogen adducts decrease in lung DNA while others increase as a consequence of enzyme induction.

The conclusion that a genetic component is involved in the etiology of lung cancer is consistent with recent molecular studies on CYP polymorphisms and lung cancer risk: In one three-generation family of 15 individuals, the high *CYP1A1*-inducible phenotype cosegregated with a polymorphic site downstream from the *CYP1A1* gene (*30*). An association between this polymorphism and increased risk for tobacco-associated squamous-cell lung cancer was reported in a Japanese population (*31*), but not in a Norwegian population (*32*). Interestingly, the susceptible *CYP1A1* genotype contracted squamous-cell carcinoma with a lower number of cigarettes than other genotypes (*33*). In addition, an association was found between restriction fragment length polymorphism in the *CYP2E1* gene and susceptibility to cigarette-related lung cancer (*34*), possibly reflecting variations in the metabolic activation of carcinogenic nitrosamines known to be present in tobacco smoke.

These results indicate that reliable phenotyping and genotyping assays should be developed and applied and justify detailed studies on *CYP1A1* gene in order to identify high-risk subjects for lung cancer among smokers. Such people once identified might be convinced to quit their habit or offered an intensive, personalized smoking cessation program.

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